Measurement of Prompt DNA Double-Strand Breaks in Mammalian Cells Without Including Heat-Labile Sites. Results for Cells Deficient in Non-homologous End Joining.

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ABSTRACT

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Ionizing radiation induces prompt single-strand breaks and double-strand breaks in DNA. In addition, labile sites are induced that can convert to breaks by heat or mild alkali. When such labile lesions are present within multiply damaged sites, additional double-strand breaks can form. Current protocols for measurement of DNA double-strand breaks involve a lysis step at elevated temperature, and consequently breaks from heat-labile sites will be generated during lysis and will be included in the measurement. However, such sites may not develop into breaks within the cell and may not need DNA double-strand break repair processes for elimination. We present here a new lysis and pulsed-field gel electrophoresis protocol that is carried out entirely at 0-4°C and thus avoids inclusion of heat-labile sites into the measurement. The new recommended lysis procedure involves two steps: the first step includes proteinase-K, which has sufficient activity at 0°C to support lysis, and the second step includes a high salt buffer to strip the DNA from histones and other proteins. By various tests we conclude that lysis is sufficient with this procedure to allow accurate determination of double-strand breaks by pulsed–field gel electrophoresis. Using the new protocol, it was found that heat-labile sites account for 30% of the initial number of double-strand breaks measured by conventional protocols after low LET radiation. In addition we show that heat-labile sites that can convert to double-strand breaks are repaired with fast kinetics and are nearly completely eliminated after 1 hr at 37°C. A study of cells deficient in non-homologous end joining reveals that the residual fast repair response typically seen in such cells is solely due to repair at heat-labile sites and is not due to repair of prompt DSBs.

INTRODUCTION

Pulsed-field gel electrophoresis (PFGE) is currently the main method used for measurement of DNA double-strand breaks (DSBs) in mammalian cells. The method involves lysis of cells in agarose plugs typically at 50°C for 17 hrs or longer in a buffer containing EDTA (0.1-0.5M), sarkosyl (1-2%), and proteinase-K (0.5-1 mg/ml) *(1-3)*. This is followed by measurements of DNA released from the agarose plug during electrophoresis (FAR, Fraction of Activity Released) with the assumption that the size of the DNA is the major determinant for release from the plug into the gel *(4)*. Alternatively, the DNA is size-fractionated in the pulsed-field gel, and the weight-fraction of DNA below a certain defined size limit is measured *(5, 6)*. The standard lysis procedure was originally developed by Blöcher and Kunhi *(7)* who showed FAR values that increased with lysis times at 50°C followed by a stable value at 17 hrs or longer. This was interpreted as a gradual release of free DNA from the cells, and a lysis time of at least 17 hrs at 50°C was recommended and is still the standard. Since ionizing radiation induces heat-labile sites that convert to DSBs at elevated temperatures *(8, 9)*, the standard warm-lysis procedure at 50°C will include those sites in the measurement *(10)*. This might give a false picture of a fast repair component of DSBs that actually corresponds to repair of heat-labile sites or repair of single-strand breaks opposite heat-labile sites *(10)*.

The presence in irradiated DNA of alkali-labile sites and heat-labile sites that can convert into single-strand breaks *(11-14)* or convert into DSBs *(8-10, 15)* is well known, although the chemical nature of the lesions is not yet well characterized. However, the most likely lesions responsible for the heat-labile sites consist of oxidative damage to the deoxyribose moiety of DNA *(10, 16, 17)*. The presence of such lesions within multiply damaged sites in DNA will cause extra DSBs at elevated temperatures. In DNA irradiated in solution the number of DSBs more than double as a result of post-irradiation heat treatment, while in intact cells the increase seems to be more moderate *(10)*. The term "prompt breaks" has been used to describe breaks present immediately after irradiation and "total breaks" to describe all those present after the labile sites are converted to breaks. It should be pointed out that the heat-labile sites may not develop into DSBs *in vivo* if they are repaired sufficiently quickly *(10)*.

The repair kinetics of radiation-induced DSBs in mammalian cells typically has a fast and a slow component *(18-22)*. The fast component has a half-life of 10-30 min and the slow

component a half life of several hours. Cells deficient in non-homologous end joining (NHEJ), either by defective Ku70, Ku80 or DNA-PKcs proteins, have a reduced fast component *(22),* leading to inefficient repair overall. However, a significant residual fast repair component remains in these cells when analyzed with conventional PFGE methods *(22-28),* which could be taken to imply the presence of an additional NHEJ-independent fast DSB repair pathway.

Here we describe the development of a method to measure prompt DSBs without including heat-labile sites. The method is carried out fully at temperatures of 0-4°C. By various means we show that lysis and release of DNA is complete with the new cold-lysis protocol. Using the new method, we show that the fast repair component seen in cells deficient in non-homologous end joining is totally due to repair of heat-labile sites and is not due to repair of prompt DSBs.

MATERIALS AND METHODS

Cell culture and irradiation

Low passages of normal human skin fibroblasts GM 5758 (Human Genetic Mutant Cell Repository, Camden, NJ) were cultured in 25 cm^2 flasks and ^{14}C-labeled as described elsewhere *(29, 30)*. The M059-K and M059-J cell lines were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured as monolayers in Dulbecco´s modified Eagle´s medium (Biochrom KG, Berlin, Germany) and Ham´s F12 nutrient mixture (Biochrom KG) in 1:1 ratio, supplemented with 10 % fetal bovine serum (Sigma) and non-essential amino acids. Normal human GM38 fibroblasts (Human Genetic Mutant Cell Repository) were cultured in McCoy's 5A medium supplemented with 10% fetal calf serum, 2 mM glutamine, 10 mM HEPES and antibiotic-antimycotic solution (GIBCO). The cells were split 1:4 and then grown to confluency for 5-7 days in the presence of 0.05 μCi/ml (1.85 MBq/l) ^{14}C-thymidine (Amersham Pharmacia Biotech) with a change to fresh medium without label the day before the experiment. Chinese hamster *xrs6* cells were maintained in exponential growth in α-MEM (GIBCO) with the same supplements as for McCoy's 5A medium. Labeling was with 0.05 μCi/ml (1.85 MBq/l) ^{14}C-thymidine for 3 days with a change to fresh medium without label 12 hr before experiments. Cells were irradiated with ^{60}Co-photons as described previously *(30)* or with ^{137}Cs-photons (Gammacell 40 Exactor, MDS Nordion, Kanata, Canada) at a dose-rate of 1.3 Gy per minute. In

experiments carried out in Berkeley, irradiations were with 320 kVp X-rays (Pantak) filtered through 0.5 mm Cu at 1.5 - 3 Gy/min. All irradiations were carried out at 0°C in medium or PBS. In experiments involving repair, cell culture flasks were quickly heated to 37°C to accurately measure the initial fast repair component.

Preparation of cells in agarose plugs

All lysis protocols were performed on cells within solidified agarose plugs (20-50 µl each). Confluent GM5758 cells were trypsinized at 37ºC and mixed with low-gelling point agarose (InCert, BMA, Rockland, ME, USA) giving a final concentration of 1.5-2.5 x 10^6 cells/ml in 0.6 % agarose in serum free medium. For analysis of DNA rejoining, GM5758 or M059 cells were irradiated as monolayers in 35 mm petri dishes as previously described *(30)*.

Irradiated GM38 or *xrs6* cells were trypsinized at temperatures below 20°C and suspended in ice-cold PBS (phosphate-buffered saline) at a concentration of 4-10 x10^6 cells/ml. The cells were then mixed with an equal volume of 1.5% low gelling temperature agarose (Sigma, type VII) in PBS and cast into plugs in a plugformer (Bio-Rad), producing plugs with size 1.2x9x20 mm. After gelling at 4°C, each plug was cut into 50 µl pieces containing 2-5x10^5 cells each.

DSBs induced by restriction enzyme.

Induction of DSBs by the restriction enzyme *HaeIII* in permeabilized cells were carried out as described by Flick *et al. (15)*. Agarose plugs with GM38 cells were incubated in permeabilization buffer (50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 50 mM NaCl, 0.5% NP-40) containing various amounts of *HaeIII* restriction enzyme. Incubation was for 1h at 0°C followed by 15 min at 30°C. Cells permeabilized without restriction enzyme were also X-irradiated at 0°C for comparison.

Lysis protocols

Seven different lysis protocols were used as summarized in TABLE 1. Plugs (20-50 µl) with embedded cells were incubated in 0.5-1 ml lysis solutions (20x plug volume) for the times and temperatures given in TABLE 1 (if not otherwise stated in the Figure legends). In experiments when plugs with cells were treated with lysis solution prior to irradiation, they were washed after

lysis for at least 6 x 1 hour in ice-cold phosphate buffer (8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 133 mM KCl, 0.8 mM $MgCl_2$ at pH 7.4) before irradiation.

Pulsed-field gel electrophoresis (PFGE)

Two different electrophoresis protocols (*Uppsala* and *Berkeley*) were used.

Uppsala. Plugs were washed 2x 1hour in 0.1 M EDTA (15x plug volumes) and 1x 1hour in 0.5x TBE at 0ºC prior to electrophoresis. The plugs were then loaded into wells in a chilled (4ºC) agarose gel (0.8% SeaKem Gold, BMA). The gel was placed into a PFGE unit (Gene Navigator, Amersham Pharmacia Biotech, Uppsala, Sweden) with 120° between the fields. DNA was separated by PFGE at 2 V/cm in TBE x 0.5 using five different phases (10, 20, 30, 40 and 60 minute pulses for 3, 5:20, 8, 9:20 and 20 hours respectively; total time 45 hours and 40 minutes). The temperature during electrophoresis was either 4°C or 11ºC. Following electrophoresis, the gels were sliced at the position of the 5.7 Mbp chromosome from *S. pombe* (BMA), and ^{14}C in the gel segments was measured by liquid scintillation. The fraction of radioactivity corresponding to DNA of size less than 5.7 Mbp is presented in text and figures as FAR < 5.7 Mbp.

Berkeley. Gel plugs treated with lysis protocols 1B or 5B were washed twice in TE (10 mM Tris, 1 mM EDTA, pH 8) at 0°C and loaded into cooled (0°C) 0.75% agarose gels in 0.5xTBE. Pulsed-field gel electrophoresis was carried out essentially as described previously *(10)* using a CHEF unit equipped with a Cooling Module (Bio-Rad) except that the temperature during the run was set to 4°C and the length of the run was increased by 50% to compensate for a slower migration rate at 4°C compared to 14°C. The switch time was 1 hr for a total run time of 30 hrs at 1.5 V/cm. Under these conditions, high molecular weight DNA up to a size of approximately 10 Mbp is eluted from the plug in the form of a compression band *(1, 4)*. After electrophoresis, the gels were cut into slices comprising the plug and the compression band and the fraction of the radioactivity present in the compression band in each lane (FAR) was measured by liquid scintillation. For more details, see *(10)*.

Protein content

The relative protein content was estimated in the GM5758 cells by measuring radioactivity from a cocktail of incorporated ^{14}C-labeled amino acids. Briefly, cells were incubated for 6-7

days with 10-20 kBq/ml labeled amino acids ([U-^{14}C]Protein hydrolysate, Amersham Pharmacia Biotech). Following 3-4 days of chase, cells were mixed with agarose as described above. Solidified agarose plugs with labeled cells were either placed directly (for protein content in intact cells) into scintillation vials or incubated in the different lysis buffers. Following 5 washes of ≥1 h in PBS, plugs were placed in scintillation vials, agarose was melted in 1 ml of 0.2 M HCl and scintillation liquid was added. In each experiment 3-6 plugs were used for each lysis protocol. For visualization of protein content, some plugs were also placed on a gel bond film (FMC), dried overnight at room temperature followed by exposure on a storage screen (Packard, Meriden, CT, USA) and evaluation on a storage phosphor system (Cyclon, Packard).

RESULTS

Ionizing radiation induces both prompt DSBs and heat-labile sites (HLS) that can convert to DSBs at elevated temperatures. Since conventional PFGE techniques include both of these lesions *(10),* we initiated a study with the aim to establish a lysis protocol that does not convert HLS into DSBs but still generates protein-free DNA that migrates normally during pulsed-field gel electrophoresis (PFGE). Obviously the temperature needs to be low, and four different cold-lysis protocols (TABLE 1, protocol 2-5) were tested in the Uppsala laboratory and compared to a standard protocol with lysis at 50°C (protocol 1). In addition, two protocols that were slight variations to protocol 1 and 5 were tested in the Berkeley Laboratory (protocols 1B and 5B). These experiments were carried out predominantly with confluent human primary fibroblasts, which were nearly exclusively (>95%) in the G1 and G0 phases of the cell cycle.

Test of various lysis solutions on irradiated intact cells

Human fibroblast cells GM5758 embedded in agarose plugs in serum free medium were irradiated with 46 Gy of ^{60}Co photons and then lysed according to lysis protocol 1-5. The DNA was then subjected to PFGE**. Figure 1** shows fraction of DNA <5.7 Mbp released by PFGE (FAR < 5.7 Mbp) for the five different treatments. Conventional lysis at 50ºC (Protocol 1) released more than 50% of the DNA whereas lysis protocol 3 was the least efficient releasing only about 5%. The difference between protocol 2 and 3 shows that proteinase-K is an important ingredient at lysis at 0°C. Using a calorimetric proteinase assay (PDQ assay from Athena

Environmental Sciences, Inc), we tested the proteinase-K activity in the lysis buffer at temperatures between 0 and 50°C. We found a temperature dependence corresponding to an activation energy of 12 kcal/mol (data not shown). The difference in activity at 0°C versus 50°C is 32-fold. Since the proteinase-K is present in large excess in the lysis buffer, its presence at 0°C results in significant proteolytic activity. However, comparison of protocols 2 and 5 shows that the DNA was not fully released by the ESP lysis buffer alone at 0°C since further treatment with a high salt buffer (protocol 5) resulted in more DNA entering the gel.

Protein content

For proper separation of DNA in pulsed-field gels it is necessary to release the DNA from proteins as well as other cellular residues that may affect the mobility during electrophoresis. To test the capacity of the lysis solutions to release cellular proteins, the amount of remaining protein content (relative to intact cells) was measured by using ^{14}C-labeled proteins **(Figure 2 and Table 2)**. Warm lysis with proteinase-K (Protocol 1) and the combined cold lysis (Protocol 5) released almost all proteins, and only around 1% was left. Protocol 2 was also relatively effective in releasing proteins, whereas the cold lysis without proteinase-K (Protocol 3) and the high-salt buffer (Protocol 4) were ineffective and left 12% and 16% residual proteins, respectively.

Irradiation of DNA from lysed cells

Proteins bound to, or associated with, DNA act as effective scavengers of radiation-induced radicals. Irradiation of completely naked DNA therefore results in DSB yields that are many times higher than those obtained in intact cells *(31).* To test the efficiency of the five different lysis protocols in producing naked DNA, intact cells in agarose plugs were lysed according to protocol 1-5, extensively washed in a low scavenging phosphate buffer and then irradiated with 0.75 Gy. The plugs were then directly analyzed by PFGE without any further treatment. The results presented in **Figure 3** show that protocol 1 and 5 were equally effective, and this further suggests that protocol 5 generates DNA without any association or attachment to protein structures. Protocol 4 was also relatively effective although this treatment keeps the attachment of DNA to the nuclear matrix intact *(32).* The other two treatments (protocol 2 and 3) seemed less effective in releasing free DNA.

Heat labile sites versus incomplete lysis

To meet the criteria of an effective lysis without transforming HLS into breaks, subsequent treatment with traditional lysis buffer, e.g. ESP buffer at 50ºC, should not lead to an increased yield of DSBs compared to exposure to heat alone. To test this, we next performed an experiment where intact cells in plugs were irradiated with photons, treated with lysis protocol 5 (ESP buffer 0ºC + HS buffer 0ºC) followed by extensive washes and incubation in either EDTA at 50°C or ESP buffer at 50ºC (protocol 1) for various times. As seen in **Figure 4** there was a clear increase of FAR < 5.7 Mbp with time at 50ºC but there was no significant difference between the two different treatments. This is further evidence that the lysis according to protocol 5 was complete and that the increase in yield with time was solely due to the conversion of HLS into DSBs.

Influence of lysis times

The time-dependence of the first step in protocol 5 was tested by varying the time in ESP buffer (at 0ºC) before the solution was changed to HS buffer (step 2). GM5758 fibroblasts were irradiated with 40 to 46 Gy photons, and the agarose plugs with cells were transferred to 0ºC ESP buffer for different times followed by HS buffer for 20-40 hours. The maximum amount of FAR <5.7 Mbp was normalized to 100%. As shown in **Figure 5** there was a large increase (from 60% to about 80%) in yield by only dipping the plug in ESP (0ºC) for a few seconds (<10 s) before the second step in salt. Following this immediate increase the curve reached maximum level (100%) within a few hours, and there was no further increase up to 45 hours. This shows that the protocol is robust in the use of an over-night incubation in the first lysis solution. We also tested the second step and found identical results for incubations between 10 and 40 hr (data not shown).

Temperature dependence for conversion of HLS to DSBs

To test the effect of post-irradiation temperature on DNA from cells lysed with protocol 5, intact GM5758 fibroblasts were irradiated in agarose plugs with 46 Gy photons. Following lysis according to protocol 5, plugs were washed and then incubated in 0.5 M EDTA at different temperatures. The data in **Figure 6** show that there was no apparent effect of incubation at 11°C

or 20ºC up to 25 hours. Further increase in temperature led to a significant increase in the amount of DNA entering the gel. This again shows that the protocol is robust in the sense that the temperatures used for lysis and PFGE are conservative.

DSBs induced by a restriction enzyme

Since restriction enzymes induce DSBs but not HLS, the results for restriction enzyme-treated cells should not depend on lysis protocol. We tested this by treating permeabilized cells in agarose plugs with *HaeIII* enzyme and then using lysis protocols 1B (50°C) or 5B (0°C). Permeabilized cells were also X-irradiated for comparison. **Figure 7** shows that FAR values were virtually identical for the enzyme–treated cells regardless of lysis protocol, while they were strikingly different for the X-irradiated cells due to the presence of HLS. This further confirms that lysis was complete in protocol 5B and that DNA was released as effectively as with conventional warm-lysis procedure. The difference seen between temperatures with X-rays in the permeabilized cells **(Fig 7, panel A**) is more pronounced than we see with intact cells and is similar to the difference previously seen with naked DNA *(10).* The reason why the relative proportion of HLS is smaller in intact cells than in naked DNA is presently not understood.

Rejoining in repair-proficient cells

Conversion of heat labile sites into DSBs during the lysis step could lead to an incorrect description of DSB rejoining. As shown in Figure 1, a standard lysis procedure (Protocol 1) at 50ºC includes heat labile sites that may add up to 40% more DSBs than the prompt number of DSBs after low-LET irradiation. To find out how quickly the heat labile sites (or associated single-strand breaks) are removed by the repair machinery, protocol 1 and 5 were used after irradiation of GM5758 cells with 40 Gy of γ-rays followed by repair incubation for 0-2 hours. The results in **Figure 8** show a clear difference between the two protocols for repair times less than 0.5 hours after irradiation followed by identical rejoining curves after more than one hour. Apparently the HLS only affect the fast kinetics of DSB rejoining after low-LET irradiation.

Experiments were also carried out comparing protocol 1B and 5B using confluent GM38 human fibroblasts. As expected, the initial yield was different for the two lysis protocols due to HLS, and this difference was absent for cells that had been incubated for 2 hours before analysis

even after high doses up to 120 Gy (data not shown). Again, this shows that HLS are quickly and efficiently repaired.

Lack of a fast rejoining component in cells deficient in non-homologous end joining (NHEJ)

Cells deficient in NHEJ have previously been depicted as partly maintaining a fast repair component that repairs as much as half of the DSBs initially present *(22-28).* We investigated to what extent this component is due to repair of HLS and to what extent it is due to repair of prompt DSBs. These experiments were carried out with growing cells distributed over all phases of the cell cycle. The human DNA-PKcs deficient fibroblast cell line M059J and control M059K from the same individual *(33)* were irradiated with 40 Gy photons and incubated for various times at 37°C for repair. DSBs were then measured using protocol 1 (warm lysis) or protocol 5 (cold lysis). As shown in **Figure 9A**, conventional lysis reveals a DNA repair defect in the DNA-PKcs deficient cell line, as seen before, but with a substantial fast repair component still present. In contrast, when the cold-lysis protocol is used (**Figure 9B**), no significant fast repair component is seen, indicating the absence of prompt DSB repair with fast kinetics.

To further verify this result for cells deficient in NHEJ, Chinese hamster *xrs6* cells deficient in Ku80 were irradiated with X-rays at a dose of 60 Gy, incubated at various times for repair and analyzed for DSBs using lysis protocols 1B and 5B. As shown in **Figure 9C**, warm lysis yielded results implicating the presence of a fast repair component while measurement with the cold-lysis protocol (**Figure 9D)** did not show any fast repair component in agreement with the results for the M059J cells. We conclude that the fast repair component consists entirely of HLS repair that is independent of DNA-PKcs and Ku80.

In these experiments with cycling cells we unexpectedly observed more DNA being released from the plug using the cold-lysis protocol compared to the warm-lysis protocol for repair times longer than 30 min (compare Figure 9A and B and Figure 9C and D). Since S-phase DNA is partly trapped in the plug using conventional lysis procedure *(20, 34-36)* we hypothesize that this could be due to different behaviour of irradiated S-phase DNA for warm and cold lysis protocols. This is presently under investigation.

DISCUSSION

Double-strand breaks are probably the most significant lesions induced by ionizing radiation, and their correct measurement and quantitation is of obvious importance. In particular when one studies the properties of cells deficient in repair pathways, the inclusion of lesions other than DSBs in the assay is a disadvantage and can lead to incorrect conclusions. We have pointed out previously that heat-labile sites (HLS) convert into DSBs during the lysis procedure and are included in PFGE assays using standard lysis conditions at 50°C *(10)*. The development of a lysis procedure that excludes the HLS is complicated by the possibility that the reduced yield in DSBs seen is partly due to inefficient lysis and not only due to the exclusion of HLS. In the present work we have tested lysis procedures carried out totally at temperatures of 0-4°C and developed a two-step procedure that combines sarcosyl and proteinase-K in the first step with a high salt extraction in a second step. The new procedure has been tested in various ways to ascertain that lysis is complete and DSBs are accurately estimated. The new cold-lysis procedure was also tested for robustness by changing parameters such as time and temperature to verify that the results are not overly sensitive to such changes. By excluding HLS from the DSB measurement, the initial yields (t=0 hours) were reduced by 30% in all three human cell lines, indicating a typical yield of 25 DSB Gy^{-1} in a diploid mammalian cell using the new lysis procedure.

The present work was carried out in two different laboratories using somewhat different protocols. We do not believe these differences are relevant to the new procedure, but for the sake of accuracy the differences are clearly indicated, and for each experiment it is implicated in which laboratory the experiment was performed. The only difference that may not be trivial is the composition of the high salt buffer used in the second step of the lysis protocol. The Uppsala procedure includes $MgCl_2$ in this buffer whereas the Berkeley procedure does not. According to our results, $MgCl_2$ does not seem to be needed when the protocol is used as indicated. It is likely that the second step is a simple high salt extraction that requires a high concentration of mono-valent salts as the active ingredient. However, the question of $MgCl_2$ is not trivial since we have observed that when the two steps are reversed, with the high salt extraction first and the proteinase-K buffer second, the $MgCl_2$ is needed in the first step for complete lysis (data not

shown). However, we have not tested this reversed procedure extensively and do not recommend it without further validation.

The new procedure is simple, and the only difference that may be inconvenient for some users is the need to run the pulsed-field gels at 4°C. However, according to the results shown in Figure 6, the heat-labile sites are rather stable in temperatures below 20°C and we expect that results will be very similar if the pulsed-field gels are run at 11°C or 14°C.

We find that cells deficient in non-homologous end-joining do not have a residual fast repair component when tested with the new protocol. This is contrary to what is seen with the conventional warm-lysis procedure. The most likely explanation is that heat-labile sites are not converted to DSBs *in vivo* and therefore do not need the NHEJ mechanism for repair. These results exclude the need to postulate a fast process for DSB repair that acts independently of NHEJ. We tentatively conclude that any NHEJ-independent DSB repair process postulated to operate in mammalian cells for example by homologous recombination would have to be a slow process. In fact, our results do not support any fast repair process for DSBs in NHEJ-deficient cells. A possible caveat to this conclusion is that cells distributed over the cell cycle were used in these experiments and the results therefore reflect an average behavior, not excluding the possibility that different processes are operating in G1, S and G2 phases. The rejoining results for the normal human fibroblast cells GM5758 and M059K indicate that both the fast and the slow rejoining components are still present when the heat-labile sites are excluded, whereas the contribution by the fast component is substantially reduced.

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REFERENCES

1. D. Blöcher, M. Einspenner and J. Zajackowski, CHEF electrophoresis, a sensitive technique for the determination of DNA double-strand breaks. *Int. J. Radiat. Biol.* **56**, 437-448 (1989).

2. D. T. Stamato and N. Denko, Asymmetric field inversion gel electrophoresis: A new method for detecting DNA double-strand breaks in mammalian cells. *Radiat. Res.* **121**, 196-205 (1990).

3. D. D. Ager, W. C. Dewey, K. Gardiner, W. Harvey, R. T. Johnson and C. A. Waldren, Measurement of radiation-induced DNA double-strand breaks by pulsed-field gel electrophoresis. *Radiat. Res.* **122**, 181-187 (1990).

4. D. Blöcher, In CHEF electrophoresis a linear induction of dsb correspond to a nonlinear fraction of extracted DNA with dose. *Int. J. Radiat. Biol.* **57**, 7-12 (1990).

5. B. Stenerlow, J. Carlsson, E. Blomquist and K. Erixon, Clonogenic cell survival and rejoining of DNA double-strand breaks: comparisons between three cell lines after photon or He ion irradiation. *Int. J. Radiat. Biol.* **65**, 631-639. (1994).

6. B. Cedervall, R. Wong, N. Albright, J. Dynlacht, P. Lambin and W. C. Dewey, Methods for the quantification of DNA double-strand breaks determined from the distribution of DNA fragment sizes measured by pulsed-field gel electrophoresis. *Radiat. Res.* **143**, 8-16. (1995).

7. D. Blöcher and M. Kunhi, DNA double-strand break analysis by CHEF (clamped homogeneous electrical field) electrophoresis. *Int. J. Radiat. Biol.* **58**, 23-34 (1990).

8. G. D. Jones, T. V. Boswell and J. F. Ward, Effects of postirradiation temperature on the yields of radiation-induced single- and double-strand breakage in SV40 DNA. *Radiat. Res.* **138**, 291-296 (1994).

9. E. S. Henle, R. Roots, W. R. Holley and A. Chatterjee, DNA strand breakage is correlated with unaltered base release after gamma irradiation. *Radiat. Res.* **143**, 144-150 (1995).

10. B. Rydberg, Radiation-induced heat-labile sites that convert into DNA double-strand breaks. *Radiat. Res.* **153**, 805-812 (2000).

11. C. Lücke-Huhle, Biological relevance of alkali-labile sites in double-stranded DNA after gamma-irradiation. *Int. J. Radiat. Biol.* **27**, 1-6 (1975).

12. J. F. Ward and I. Kuo, Strand breaks, base release, and postirradiation changes in DNA gamma-irradiated in dilute O2-saturated aqueous solution. *Radiat. Res.* **66**, 485-498 (1976).

13. M. V. Lafleur, J. Woldhuis and H. Loman, Alkali-labile sites and post-irradiation effects in gamma-irradiated biologically active double-stranded DNA in aqueous solution. *Int. J. Radiat. Biol.* **36**, 241-247 (1979).

14. J. F. Ward, Biochemistry of DNA lesions. *Radiat. Res.* **104**, S103-S111 (1985).

15. M. B. Flick, R. L. Warters, L. S. Yasui and R. E. Krisch, Measurement of radiation-induced DNA damage using gel electrophoresis or neutral filter elution shows an increased frequency of DNA strand breaks after exposure to pH 9.6. *Radiat. Res.* **119**, 452-465 (1989).

16. C. von Sonntag and D. Schulte-Frohlinde, Radiation-induced degradation of the sugar in model compounds and in DNA. *Mol. Biol. Biochem. Biophys.* **27**, 204-226 (1978).

17. C. von Sonntag, *The Chemical Basis of Radiation Biology,* Taylor & Francis, London, 1987.

18. E. Dikomey and J. Franzke, Three classes of DNA strand breaks induced by X-irradiation and internal beta-rays. *Int. J. Radiat. Biol.* **50**, 893-908. (1986).

19. M. J. Peak, L. Wang, C. K. Hill and J. G. Peak, Comparison of repair of DNA double-strand breaks caused by neutron or gamma radiation in cultured human cells. *Int. J. Radiat. Biol.* **60**, 891-898 (1991).

20. G. Iliakis, The role of DNA double strand breaks in ionizing radiation-induced killing of eukaryotic cells. *Bioessays* **13**, 641-648. (1991).

21. B. P. Kysela, B. D. Michael and J. E. Arrand, Relative contributions of levels of initial DNA damage and repair of double strand breaks to the ionizing radiation-sensitive phenotype of the Chinese hamster cell mutant, XR-V15B. Part I. X-rays. *Int. J. Radiat. Biol.* **63**, 609-616 (1993).

22. H. Wang, Z. C. Zeng, T. A. Bui, E. Sonoda, M. Takata, S. Takeda and G. Iliakis, Efficient rejoining of radiation-induced DNA double-strand breaks in vertebrate cells deficient in genes of the RAD52 epistasis group. *Oncogene* **20**, 2212-2224. (2001).

23. L. M. Kemp, S. G. Sedgwick and P. A. Jeggo, X-ray sensitive mutants of Chinese hamster ovary cells defective in double-strand break rejoining. *Mutat. Res.* **132**, 189-196 (1984).

24. J. Dahm-Daphi, E. Dikomey, C. Pyttlik and P. A. Jeggo, Reparable and non-reparable DNA strand breaks induced by X-irradiation in CHO K1 cells and the radiosensitive mutants xrs1 and xrs5. *Int. J. Radiat. Biol.* **64**, 19-26 (1993).

25. F. Chen, S. R. Peterson, M. D. Story and D. J. Chen, Disruption of DNA-PK in Ku80 mutant xrs-6 and the implications in DNA double-strand break repair. *Mutat. Res.* **362**, 9-19 (1996).

26. P. J. Johnston, S. H. MacPhail, T. D. Stamato, C. U. Kirchgessner and P. L. Olive, Higher-order chromatin structure-dependent repair of DNA double-strand breaks: involvement of the V(D)J recombination double-strand break repair pathway. *Radiat. Res.* **149**, 455-462 (1998).

27. S. J. DiBiase, Z. C. Zeng, R. Chen, T. Hyslop, W. J. Curran, Jr. and G. Iliakis, DNA-dependent protein kinase stimulates an independently active, nonhomologous, end-joining apparatus. *Cancer Res.* **60**, 1245-1253. (2000).

28. R. Okayasu, K. Suetomi, Y. Yu, A. Silver, J. S. Bedford, R. Cox and R. L. Ullrich, A deficiency in DNA repair and DNA-PKcs expression in the radiosensitive BALB/c mouse. *Cancer Res.* **60**, 4342-4345. (2000).

29. E. Höglund, E. Blomquist, J. Carlsson and B. Sternerlöw, DNA damage induced by radiation of different linear energy transfer: initial fragmentation. *Int. J. Radiat. Biol.* **76**, 539-547 (2000).

30. B. Stenerlöw, E. Höglund and J. Carlsson, Induction and rejoining of large DNA fragments after ion irradiation. *Radiat. Res.* **151**, 642-648 (1999).

31. R. L. Warters and B. W. Lyons, Variation in radiation-induced formation of DNA double-strand breaks as a function of chromatin structure. *Radiat. Res.* **130**, 309-318. (1992).

32. B. D. Nelkin, D. M. Pardoll and B. Vogelstein, Localization of SV40 genes within supercoiled loop domains. *Nucleic Acids Res.* **8**, 5623-5633. (1980).

33. M. J. Allalunis-Turner, G. M. Barron, R. S. Day, 3rd, K. D. Dobler and R. Mirzayans, Isolation of two cell lines from a human malignant glioma specimen differing in sensitivity to radiation and chemotherapeutic drugs. *Radiat. Res.* **134**, 349-354. (1993).

34. G. E. Iliakis, L. Metzger, N. Denko and T. D. Stamato, Detection of DNA double-strand breaks in synchronous cultures of CHO cells by means of asymmetric field inversion gel electrophoresis. *Int. J. Radiat. Biol.* **59**, 321-341. (1991).

35. R. L. Warters and B. W. Lyons, Induction and repair of double-strand breaks in the replicating DNA of HeLa cells. *Radiat. Res.* **134**, 337-342. (1993).

36. W. C. Dewey, R. S. Wong and N. Albright, Pulsed-field gel electrophoretic migration of DNA broken by X irradiation during DNA synthesis: experimental results compared with Monte Carlo calculations. *Radiat. Res.* **148**, 413-420 (1997).

37. J. Nygren and G. Ahnstrom, DNA double- and single-strand breaks induced by accelerated He2+ and N6+ ions in human cells: relative biological effectiveness is dependent on the relative contribution of the direct and indirect effects. *Int. J. Radiat. Biol.* **70**, 421-427. (1996).

TABLE 1. Lysis protocols.

Protocol #	Lysis conditions	Comments
1	ESP-buffer, 4°C for 1h +50°C for 18-20 h.	Normal ESP lysis (Uppsala)
1B	ESP-bufferB, 4°C for 1h, + 50°C for >20 h.	Normal ESP lysis (Berkeley)
2	ESP-buffer, 0°C for 30-40 h.	Cold ESP lysis.
3	ESP-buffer without proteinase-K, 0°C for 30-40 h.	ES=modified cold ESP lysis.
4	HS-buffer, 0°C for 30-40 h.	High Salt (HS) lysis.
5	ESP-buffer, 0°C for 20-25 h + HS-buffer, 0°C for 20-40 h.	Complete cold-lysis protocol (Uppsala).
5B	ESP-bufferB, 0°C for >20 h +1x wash in TE + HS-bufferB, 0°C for 24 h.	Complete cold-lysis protocol (Berkeley).

ESP-buffer: 0.5 M EDTA, 2% N-lauroylsarcosine,1 mg/ml proteinase-K (Roche Diagnostics, Mannheim, Germany), pH 8.0 (used in Uppsala). Proteinase-K is added just before use.

ESP-bufferB: 0.4 M EDTA, 2% N-lauroylsarcosine, 1 mg/ml proteinase-K (Gibco-Invitrogen or USB), pH 8.0 (used in Berkeley). Proteinase-K is added just before use.

HS-buffer: 1.85 M NaCl, 0.15 M KCl, 5 mM $MgCl_2$, 2mM EDTA, 4 mM Tris, 0.5 % Triton X-100, pH 7.5 (used in Uppsala). Triton X-100 is added just before use. Adopted from *(37)*.

HS-bufferB: 1.85 M NaCl, 0.15 M KCl, 4 mM Tris, 0.5 % Triton X-100, pH 7.5 (used in Berkeley). Triton X-100 is added just before use.

Table 2. Protein content after different treatments

Protocol	% protein[a] ±SD
1. ESP-buffer, 50°C	0.8 ±0.4
2. ESP-buffer, 0°C	2.3 ±1.4
3. ES-buffer, 0°C	11.9 ±5.5
4. HS-buffer, 0°C	16.2 ±1.5
5. ESP-buffer, 0°C + HS-buffer, 0°C	1.2 ±0.5

[a] Protein content, relative to that in intact cells, after treatment of GM5758 fibroblasts in different lysis buffers. Mean and standard deviation from four independent experiments.

Figure Legends:

Figure 1. Fraction of DNA <5.7 Mbp released by PFGE (FAR < 5.7 Mbp) after irradiation of intact GM5758 fibroblast cells with 46 Gy of photons followed by lysis according to five different protocols (1-5). Mean and SEM of 4-5 independent experiments.

Figure 2. Phosphoimage of ^{14}C-labeled proteins after treatment of intact human fibroblasts (GM5758) in agarose plugs with different lysis solutions. Intact cells without any lysis are shown to the left. Lane 1-5 corresponds to treatment in lysis protocol 1-5, respectively.

Figure 3. FAR <5.7 Mbp for GM5758 cells pre-treated with lysis protocol 1-5, extensively washed in phosphate buffer and then irradiated with 0.75 Gy. Mean and SEM of 5-6 independent experiments.

Figure 4. Effect of post-treatment at 50°C. GM5758 cells were irradiated with 46 Gy, lysed according to protocol 5 (ESP-buffer, 0°C + HS-buffer, 0°C), washed and then incubated in either ESP or 0.5 M EDTA at 50°C for various periods of time. Data from three independent experiments.

Figure 5. Time-dependence of protocol 5. GM5758 cells were irradiated with 40 to 46 Gy photons after which the agarose plugs with cells were transferred to 0ºC ESP-buffer for the indicated times before the solution was changed to HS-buffer (step 2). Data points were normalized to 100% (where FAR <5.7 Mbp reach maximum). Mean and SEM from 2-5 independent experiments.

Figure 6. Stability of HLS at various temperatures. Intact fibroblasts in agarose plugs were irradiated with 46 Gy photons and lysed according to protocol 5. After washing in 0.5 M EDTA, the plugs were incubated at the stated temperature for various times before PFGE. T=0 h corresponds to samples that were kept on ice during the whole incubation. Data from three independent experiments (37ºC data from single experiment). Data for 50ºC are from Figure 4.

Figure 7. Restriction endonuclease treatment. Permeabilized human fibroblasts GM38 were either X-irradiated (panel A) or treated for 15 min at 30°C with various amounts of restriction endonuclease *HaeIII* (panel B). Agarose plugs with cells were cut in half and lysed according to protocol 1B (warm lysis) or protocol 5B (cold lysis). The DNA was then analyzed with PFGE (Berkeley protocol).

Figure 8. Rejoining of DSBs in GM5758 fibroblasts irradiated with 40 Gy photons. After repair incubation at 37ºC cells were lysed according to either protocol 1 (ESP-buffer, 50ºC) or protocol 5 (ESP-buffer , 0°C + HS-buffer, 0°C). Mean and maximum deviation from two independent experiments.

Figure 9. Rejoining of DSBs in cells deficient in NHEJ as measured by warm and cold lysis protocols. Human fibroblasts M059J (DNA-PKcs deficient) and M059K (control) were irradiated with 40 Gy ^{137}Cs-photons and incubated for various times for repair. Plugs with cells were treated with protocol 1(warm lysis, panel A) or protocol 5 (cold lysis, panel B) and analyzed with PFGE (average from 3-4 experiments with standard deviation). Likewise *xrs6* cells were X-irradiated with 60 Gy and then incubated at 37°C for the times indicated. Plugs with cells were treated with protocol 1B (warm lysis, panel C) or protocol 5B (cold lysis, panel D) and subjected to PFGE (Berkeley protocol). Average and SEM from three independent experiments are shown in panel C and D.

Figure 2

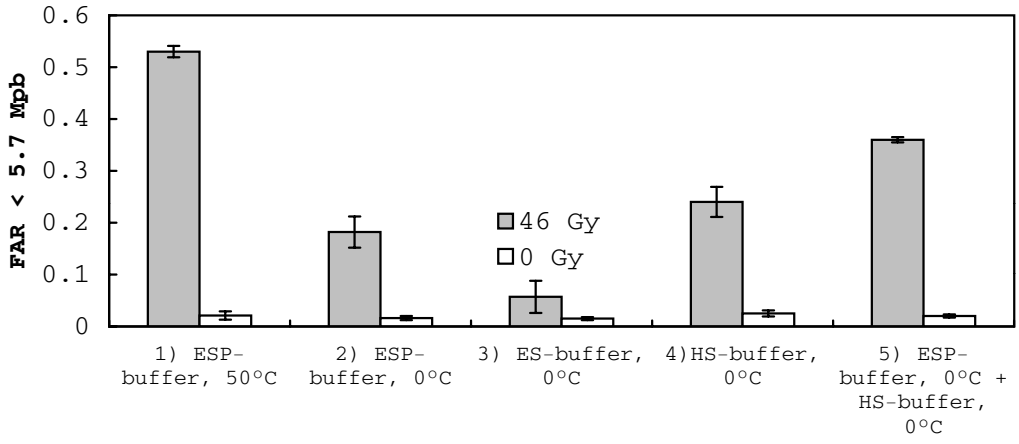

FAR < 5.7 Mpb
0.6
0.5
0.4
0.3
0.2
0.1
0
46 Gy
0 Gy
1) ESP-buffer, 50°C
2) ESP-buffer, 0°C
3) ES-buffer, 0°C
4)HS-buffer, 0°C
5) ESP-buffer, 0°C + HS-buffer, 0°C

Figure 1

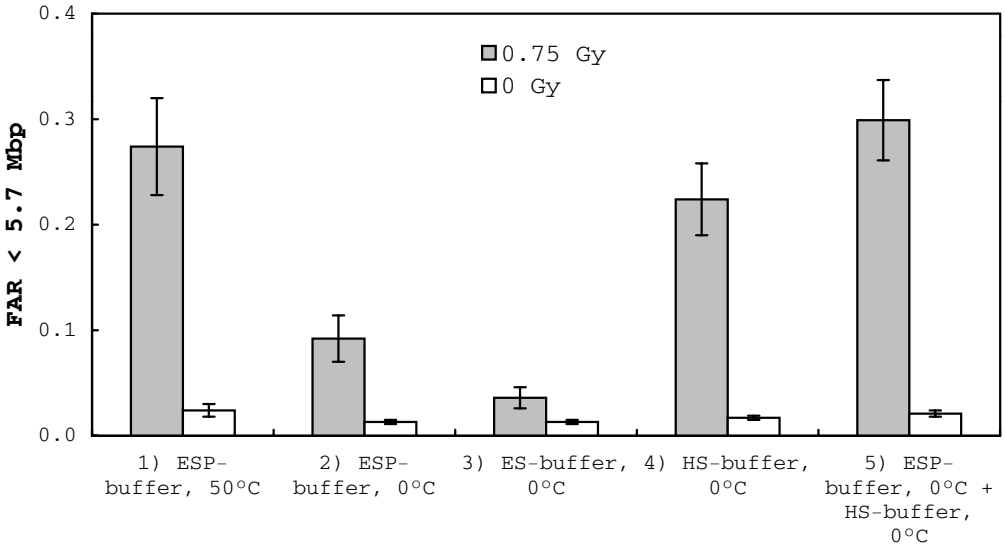

0.4
0.3
0.2
0.1
0.0
FAR < 5.7 Mbp
0.75 Gy
0 Gy
1) ESP-buffer, 50°C
2) ESP-buffer, 0°C
3) ES-buffer, 0°C
4) HS-buffer, 0°C
5) ESP-buffer, 0°C + HS-buffer, 0°C

Figure 3

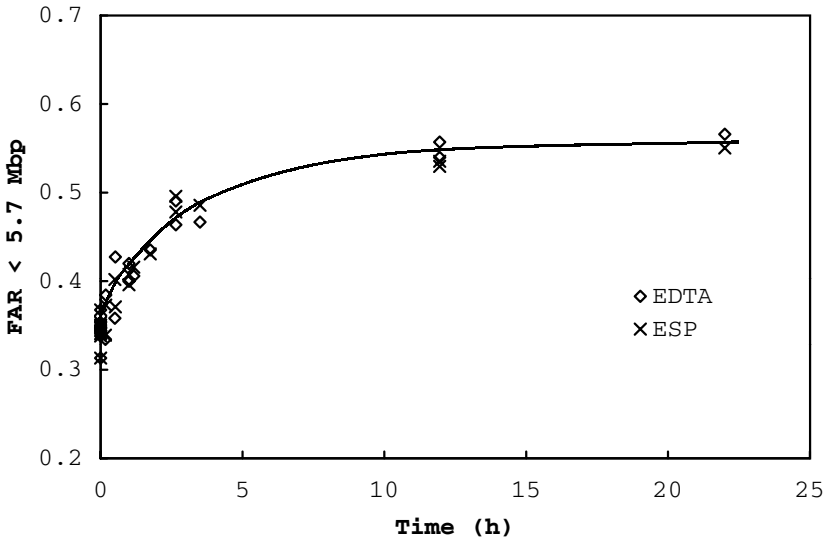


Figure 4

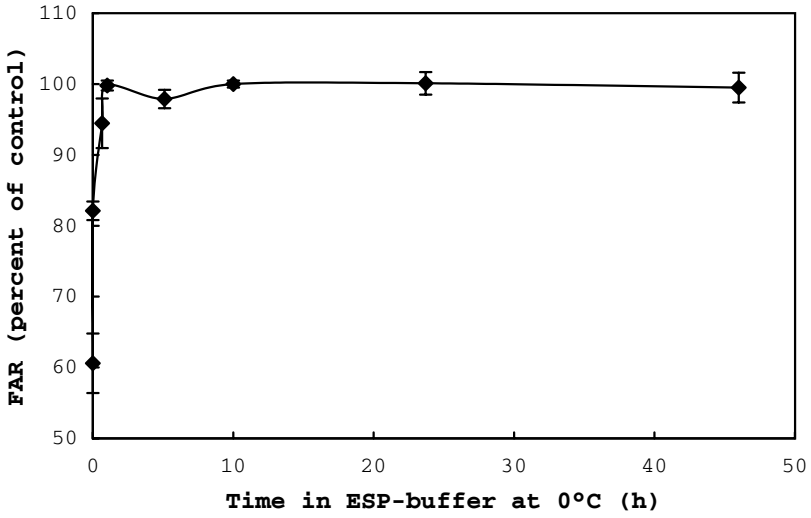

110
100
90
80
70
60
50
FAR (percent of control)
0
10
20
30
40
50
Time in ESP-buffer at 0°C (h)

Figure 5

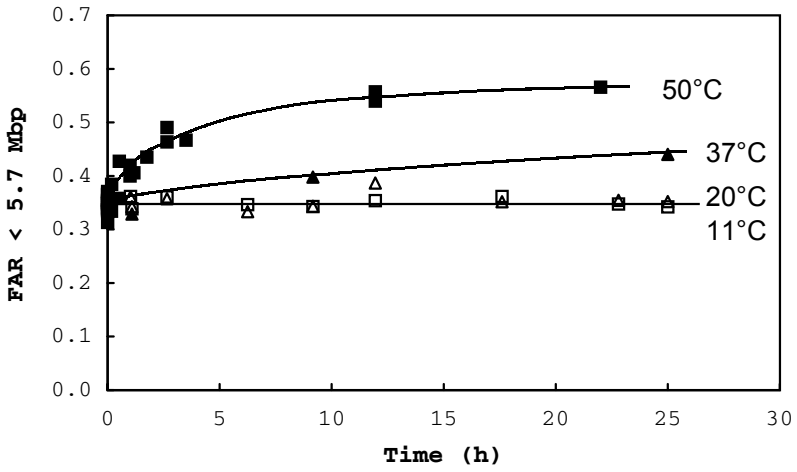

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
FAR < 5.7 Mbp
0
5
10
15
20
25
30
Time (h)
50°C
37°C
20°C
11°C

Figure 6

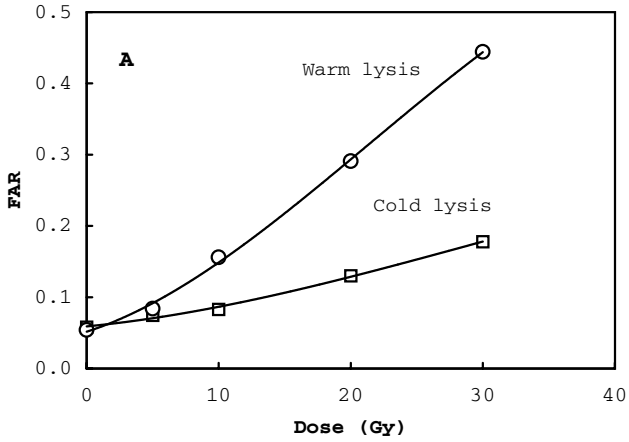


Figure 7A

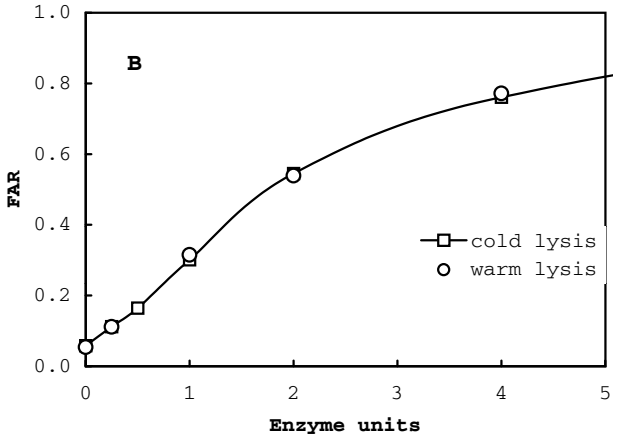
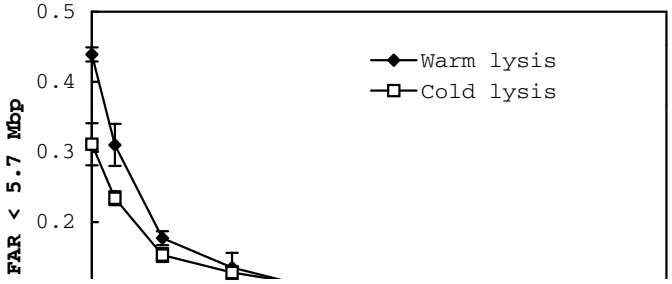


Figure 7B



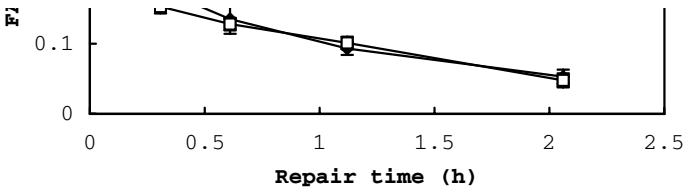


Figure 8

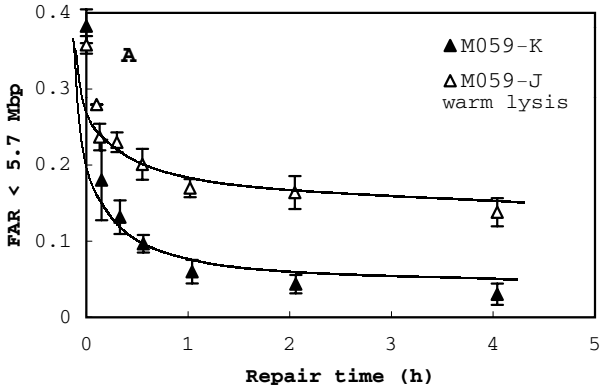


Figure 9A

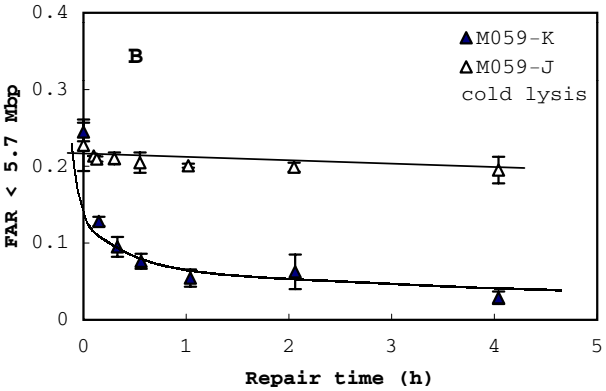
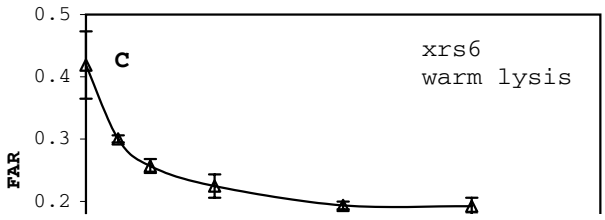


Figure 9B



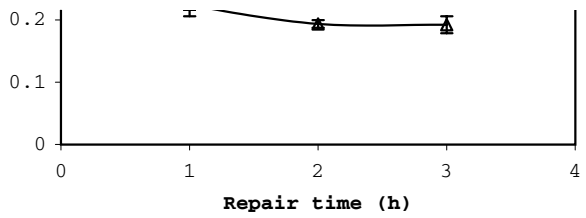


Figure 9C

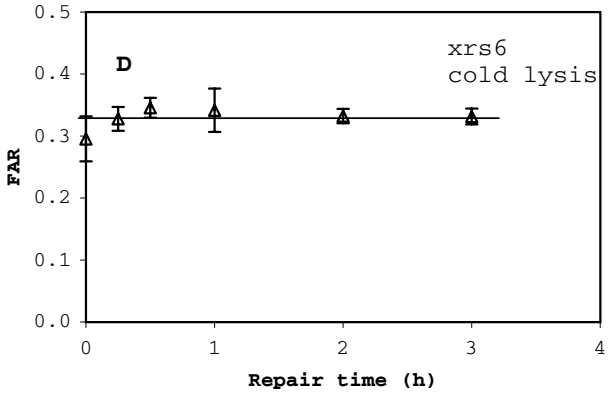


Figure 9D